IN VITRO PRODUCTION OF COLCHIPLOIDS AND MUTANTS OF NATIVE FLORIDA DIPLOID *Vaccinium* SPECIES AND HYBRIDS AND EVALUATION OF 12X *V. ASHEI* COLCHIPLOIDS

BY

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IN VITRO PRODUCTION OF COLCHIPLOIDS AND MUTANTS OF NATIVE FLORIDA DIPLOID *Vaccinium*, SPECIES AND HYBRIDS AND EVALUATION OF 12X *V. ASHEI* COLCHIPLOIDS

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In vitro produced colonies of Florida native diploid blueberry species *Vaccinium elliottii* Chapm., *V. darrowi* Camp, and their interspecific hybrid were treated with colchicine to double their chromosome number and facilitate interspecific hybridization with tetraploid highbush blueberry, *V. corymbosum* Lamarck. *In vitro* colchiploids, identified by increased shoot diameter, were confirmed as tetraploid by stomate guard cell measurement and chromosome counts from shoot tip squashes.

V. elliottii produced the greatest number of increased-diameter shoots when 2-node cuttings were treated for 48 hours on a rotary drum in liquid modified Knops medium containing 0.1% colchicine, or for 2-6 weeks without agitation on solid modified Knops medium containing 0.01% colchicine. *V. darrowi* produced the greatest number of

increased-diameter shoots when 2-node cuttings were treated on a rotary drum in .01% colchicine-containing liquid modified Knops medium for 24 hours. The V. elliottii X V. darrowi hybrid produced the greatest number of increased diameter shoots when 2-node cuttings were treated for 4 hours in colchicine-free liquid modified Knops medium on a rotary drum, apparently due to spontaneous doubling.

The LD-50 of in vitro V. elliottii colonies treated with gamma radiation was 4.5 krads. No explants receiving a dose greater than 25 krads survived. Two mutant colonies were identified from the 1.5 krad treatment and 1 mutant colony was identified from the 3 krad treatment. These mutant colonies were dwarfed and feathery in appearance.

The 12X colchiploid ramets of V. ashei cultivars 'Beckyblue' and 'Bluebelle' apparently are of low fertility as both male and female parents. Pollen germination was low and pollen tubes weak. No fruit was set when the 12X cultivars were intercrossed or crossed with 6X V. ashei 'Bonita'. A few fruit were set when the ramets were crossed with tetraploid V. corymbosum 'Sharpblue' or diploid V. darrowi, possibly due to accidental outcrossing or selfing.

CHAPTER I

INTRODUCTION

Blueberry, (Vaccinium species), is native to North America, Europe and several other parts of the world. Many wild species exist in the United States, with chromosome ploidys ranging from diploid to hexaploid. Wild relatives are valuable sources of locally adaptive characteristics which would be useful if they could be incorporated into cultivated species.

Most blueberry acreage in Florida is planted with hexaploid rabbiteye (V. ashei Reade) cultivars. This is due to the resistance of V. ashei to Phytopthora cinnamoni Rands root rot, cane canker (Botryosphaeria corticis Demaree and Wilcox) and various other fungal diseases (74, 75) and to its low chilling requirement (97), high vigor and high productivity (45). Highbush (V. corymbosum) cultivars are also widely planted in the South, but several factors impede their cultivation in Florida, including fungus susceptibility, higher winter chilling requirement and lack of heat and drought tolerance. Despite these weaknesses, V. corymbosum has one important advantage over V. ashei - early ripening. With bloom occurring at approximately the same date, fruit of early highbush clones mature in 60 days while

it takes early rabbiteye cultivars 80 to 90 days post-pollination to reach maturity (45, 95).

Florida native blueberry species include diploids (2N=2X=24) V. darrowi Camp, V. elliottii Chapm., V. atrococcum Heller, V. arboreum Marsh, and V. staminium L.; tetraploids (2N=4X=48) V. fuscatum Ait., and V. myrsinites Lam. and the hexaploid (2N=6X=72) V. ashei (45, 73). In the section Cyanococcus, which includes all of the above species except V. arboreum and V. staminium, there are only weak sterility barriers between species with the same ploidy level (8, 18). Heteroploid crosses give variable results ranging from partial success to almost complete failure. Hybrids derived from heteroploid crosses may exhibit ovule or pollen sterility (27).

Several of the wild diploid Vaccinium species native to Florida possess characteristics which could complement the tetraploid highbush genome. Diploid V. elliottii is adapted to the drier Florida soil, has high resistance to certain fungal diseases, high berry flavor, low chilling requirement and high vigor. V. darrowi has low chilling requirement, heat and drought resistance, light blue fruit color, attractive lowbush growth form and some cane canker resistance (45, 75, 96, 109). Because of a triploid block, few hybrids are obtained from diploid by tetraploid crosses and their reciprocals (4).

There are several possible methods for bypassing the triploid block. These methods include production of haploids from tetraploid V. corymbosum, rescue of triploid embryos prior to endosperm failure, enhancement of 2N gamete production in diploid species, and colchicine treatment of diploid species to convert them to autotetraploids.

Chromosome doubling of woody perennial species by colchicine treatment has had limited success (29, 37, 38, 40, 48, 56). Treatment in vitro has several advantages over traditional methods, including a stable, germ-free environment with provision of optimum light, nutrients, and humidity which allows survival of weaker autotetraploids. Shoots produced in tissue culture are very vigorous and have short internodes and numerous buds which make them highly suited for chromosome doubling with colchicine. Tissue culture is also quite space conservative, allowing large numbers of shoots to be treated in a small area (53, 82). Polyploid shoots, characterized in vitro by increased shoot diameter, can be quickly and easily screened by visual examination (72).

Mutation has played a large part in the development of fruit cultivars. Mutation induction in vegetatively propagated species can be valuable in altering a few traits in an otherwise outstanding cultivar or to induce tissue rearrangements of existing periclinal chimeras (31,41,42).

Determination of the fertility of autoploids is essential to their use in a breeding program. Not only must the

pollen germinate, it must also have the ability to effect fertilization and allow fully-developed, fertile seed to be set.

The purpose of this study was to identify the best type of explant material for blueberry chromosome doubling by *in vitro* colchicine treatment as well as optimal colchicine concentration and treatment duration for enhancement of doubling. Additional objectives were to find the optimal radiation treatment for mutant production, and to evaluate the breeding value of 12X *V. ashei* colchiploids.

CHAPTER II

LITERATURE REVIEW

Polyploidy has been an important feature of plant evolution, and its value in cultivar breeding has long been recognized. Possible beneficial changes induced by polyploidy include broader, thicker leaves, larger flowers and fruit and increased fertility of hybrids not fertile as diploids (35, 48). The evolution of the genus Vaccinium has occurred through millions of years and polyploidy has played a large part in the process (29).

Harlan and DeWet suggest that the most common and widespread form of spontaneous polyploidy in the higher plants is probably by 2N + N reproduction (52). This occurs by a two-step process. The first step consists of fusion of a 2N female gamete with a normal N male gamete giving rise to a triploid plant. This triploid plant, in turn, may produce some cytologically unreduced triploid female gametes that are fertilized by haploid gametes of the diploid parents resulting in tetraploid offspring (66). This is a general phenomenon that probably takes place at a low but significant frequency in nearly all species of sexual plants (52). Approximately 75% of all monocot species and 40% of the dicots are considered polyploid (66).

The methods used to induce polyploidy may be divided into 2 classes, somatic doubling and gametic doubling. In somatic doubling, the chromosomes which would normally be distributed to 2 sister somatic nuclei are included in 1 nucleus. If the diploid plant in which this occurs is not a hybrid, it gives rise to an autopolyploid, with 4 very similar sets of chromosomes. If the diploid plant is a wide hybrid, somatic doubling gives rise to an allopolyploid with 2 pairs of identical chromosome sets (5, 48).

In gametic doubling, the chromosomes which would have been distributed to the 4 nuclei produced by meiosis are included in 2 nuclei which give rise to diploid gametes. Most of the natural polyploids produced by this method have come from hybrids with low fertility. The zygotes which are produced generally arise from unreduced gametes, male or female or both (48).

With the discovery of the polyploidizing action of colchicine in the 1930's, polyploid induction was no longer left to nature's whim. A number of other agents, chemical and physical, have been used to induce polyploidy including brome-acenaphthene (99), temperature shock (34), severe pruning to encourage adventitious bud break (5, 56), nitrous oxide, and podophyllin (62). The greatest success in polyploid induction, however, has been with colchicine treatment (3, 11, 21, 34, 35, 40, 46, 48, 72, 80, 89).

Colchicine is a mitotic inhibitor which acts by interfering with the equilibrium between microtubules and their

subunits, preventing the assembly of spindle fibers and ultimately blocking completion of mitosis (65). Colchicine in aqueous solution is readily diffusible into plant tissue and acts over a wide range of concentrations with high specificity. The cellular reaction to colchicine depends on several conditions: specific concentration, exposure period, mitotic stage when contacted by the chemical, cell type and presence of an environment favorable to mitosis (11). Colchicine acts only on dividing cells; with no effect on resting cells (99). The mitotic stage at which colchicine is most effective in lowest concentration is late prophase (46); however, the effect of colchicine is not confined to a limited number of cells at a particular stage of cellular development. Any cell may be affected if the cell goes through division while containing the chemical (34, 35). As long as colchicine remains in treated material above threshold concentration, affected cells will repeatedly fail to divide at the end of each nuclear division cycle resulting in multipolyploidy (34). When colchicine is removed, the chromosomes gather into small groups and loose spindles appear (46). A common aberration following treatment is aneuploidy, which seems to be due to partial instead of total arrest of chromosome division or to a multipolar division in some of the polyploid nuclei (35).

Chromosome duplication may result in an increase in nuclear and cell volume. Changes in volume and cell

dimensions are often correlated with changes in size of plant parts (34). New leaves and stems which grow from doubled material are usually wrinkled, thicker, darker green, and coarser in texture. Leaf veins of doubled material become coarser, leaf hairs and glands become coarser and more abundant, leaf profile may become shorter and more rounded and flower buds produce shorter, stockier pedicels. Similarly, the petals may be stunted and coarsened in texture or larger and the buds more compact. In general, a polyploid plant has a more rugged appearance, looks sturdier and has certain giant-like features. Usually growth is slower, and the plant is usually shorter than its original diploid counterpart (5, 34, 100).

Colchicine induction of polyploidy has been successful with many herbaceous species (34, 48, 100). Woody species, however, are generally unresponsive to conventional methods of colchicine treatment. Azalea (89), camellia (3), grape (47), cranberry (36), pear (56) and chestnut (40) have been doubled with limited success, while plum, blueberry, cherry, peach and apple are very difficult species in which to induce polyploidy (40, 48).

A problem common to both herbaceous and woody species following colchicine treatment is chimera formation due to the multicellular nature of the bud apex (12). Chimeras may be periclinal or sectorial. Growing points are usually made up of 3 independent cell layers termed L-I, L-II, and L-III.

When only a single layer is affected by colchicine, a periclinal chimera results. When a portion or sector of all 3 layers is doubled, a sectorial chimera is the result. Utilization of colchiploids depends upon identification of plants which produce doubled gametes. These include solidly doubled plants and plants with a doubled L-II layer. The anticlinal mode of cell division characteristic of growing shoot tips is not a permanently fixed condition. Periclinal division occurs occasionally in cells of the L-I, more frequently in the L-II and commonly in the L-III histogenic layer with the result of varying the ploidy level of colchiploid plant layers (39).

Identification of the ploidy level of each layer is desirable. Measurement of stomatal guard cells of the epidermis of colchicine-treated versus untreated plants has been used to identify doubled L-I layers in a number of species including Vaccinium (1, 19, 20), Bromus (104), and Triticum (93). Increased intensity of green leaf color of doubled plants has been found to be associated with the increased depth of leaf tissue and a proportionate increase in number of chloroplasts (63). Chloroplast numbers in stomatal guard cells are also found in increased numbers in doubled plants and are another measurable indicator of increased ploidy of the L-I layer in some species (66, 94).

The L-II layer, which gives rise to the gametes, is of greatest interest to plant breeders. Autotetraploids have larger pollen grains than their diploid counterparts (33).

These grains may take on characteristic shapes providing a morphological indication of induced polyploidy (34).

The L-III layer gives rise to various internal tissues. Chromosome doubling of this layer may be measured by chromosome counts from root or shoot tip squashes (5, 56).

Naturally amphiploid species generally do not show consistent increases in these parameters when compared with parental species. Genetic factors and the nature of the parental species may be more influential than the change in chromosome number (46).

Various methods of increasing the effectiveness of colchicine treatments have been tested. These include pretreatment of plant material with gibberellic acid to accelerate the rate of cell division and cell elongation. This acceleration is postulated to increase cell permeability to colchicine (110). Etiolation has also been used to induce cell elongation. Exposure of etiolated shoots to light causes cell division to occur simultaneously in a large number of cells making the shoots more susceptible to the doubling action of colchicine (21, 55). Treatment of Allium root tips with 20 - 50 ppm of indole-3-acetic acid has been shown to induce mitosis in a large number of cells. Tips were treated for 4 hours and a large number of mitotic figures were recorded 24 hours later (44).

DMSO (dimethyl sulfoxide) has been used as a carrier for colchicine, increasing treatment effectiveness by increasing drug penetration (92). Other factors affecting

treatment success are pH of the colchicine solution (66), aeration of the solution (2), and treatment temperature (21).

Cytology

Subfamily Vacciniaceae is a taxonomically ancient group with many cytologically primitive characteristics. The basic karyotype of the section Cyanococcus has not evolved much from the ancestral form characterized by high chromosome number and small, metacentric chromosomes (17, 23). The basic haploid complement of Vaccinium is considered to be 12 chromosomes (2 long, 8 intermediate and 2 short) metacentrics) (51). The whole section Cyanococcus in eastern North America forms a large polyploid complex in which both autoployploidy and allopolyploidy have played significant roles (17, 18). There are naturally occuring diploid, tetraploid and hexaploid species and a few pentaploid hybrids (28). According to Camp, all but 2 diploid species (V. elliottii and V. myrtilloides) have given rise to polyploids. All species, diploid to hexaploid, have been found to produce unreduced pollen with an especially high rate in diploid species (22). These unreduced gametes allow interspecific heteroploid crosses which would not normally be possible (9, 27). Members of diploid and tetraploid groups rarely hybridize but are generally interfertile within any ploidy group (24). Barriers to natural hybrid production among homoploids include weak incompatibility

barriers within the style which slow pollen tube growth (106) and, to a limited extent, differing pollinizers (105).

Cytological studies in Vaccinium have revealed regular pairing in diploid species (68). An exception to this is a diploid hybrid between V. myrtillus X V. vitis ideaea which showed a number of meiotic irregularities due to chromosomal differences. Each parental species alone was quite regular in meiosis. An autotetraploid created by doubling the chromosome number of V. myrtillus displayed a number of multivalent associations at metaphase I (91).

Vander Kloet postulated that V. corymbosum originated from an ancient hybrid swarm between V. tenellum X V. darrowi (107). Bivalents only were found in some clones of V. corymbosum (60, 68), with multivalence and secondary pairing indicative of segmental allopolyploidy occurring in others (59, 83). Trivalents and univalents were rare. The high frequency of bivalents may be due to the almost complete diploidization of the tetraploid species (60). Most of the bivalents are involved in pseudo-multivalent associations. Highbush cultivar 'Jersey' produced bivalents and quadrivalents in meiosis with 0-50% of the chromosome complement participating in quadrivalent associations (59, 60). The primary factors governing the type and frequency of multivalent association in polyploids are chiasma frequency and position, size of chromosomes (61), and genes that regulate pairing.

Tetraploid V. uliginosum, possibly due to its presumed recent autotetraploid origin, has a number of meiotic irregularities. When V. uliginosum was crossed with cultivated highbush, meiosis was quite regular due perhaps to autosyndetic pairing (91).

Hexaploid blueberry species studied by Longley included V. ashei, V. constablaei and V. amoenum and were generally found to be meiotically stable (68).

Throughout the plant kingdom, autoploids are almost without exception less fertile than diploids. This is probably due to multivalent associations, which result in unbalanced gametes. Great variations in fertility are found from species to species, ranging from almost complete sterility to 75% fertility (87).

Among progenies of alloploids, the first generation may be quite fertile while later generations have reduced fertility (46).

In a study where pollen germination was used as an indicator of fertility of Vaccinium species and hybrids, tetraploid V. corymbosum pollen germination ranged from 22-49%, tetraploid V. myrsinites pollen averaged 42% germination and 27% of diploid V. darrowi germinated (49). Non-functional pollen is characterized by tetrad collapse and large globules of sporopollenin deposit.

A second fertility indicator is seed set (108). Four types of seed have been described in blueberry fruit: large, well-filled seed with brown or tan seed coat; large

flattened or concave brown or tan seed which may be partially shriveled; well-filled brown or tan seed half the size of class 1; or very small white seed which appear empty. Only the seeds of the first class are fully developed and capable of germination (7, 25). Among highbush cultivars, only 21-52% of the seeds were found to be fully developed, while 36-55% of open-pollinated rabbiteye seeds were judged to be capable of germination (79).

Improvement

Blueberry improvement was begun by Coville with a species cross of wild tetraploid V. corymbosum clone 'Brooks' with V. angustifolium clone 'Russell' (24). Northern tetraploid cultivars lack several important characteristics which would make them optimal southern cultivars (56, 73, 75, 77). Problems include a high chilling requirement and susceptability to cane canker and root rot fungal diseases. The characteristics of the Vaccinium species native to Florida are listed in Table 1. As can be seen in the table, some native Florida, diploid species are disease resistant, vigorous, and tolerant to Florida soils and climate (73, 74, 76). Crosses between these 2 ploidy levels are unsuccessful due to developmental failure of triploid seed (4, 91). One way to combine the favorable characteristics of the 2 ploidys is to equalize their chromosomes numbers by colchicine treatment of diploid species to produce autotetraploids (78,87,89).

Table 1

Ploidy Level and Characteristics of *Vaccinium* Species Native to Florida

Ploidy	Species	Possible desirable characteristics
2N=6X=72	*V. ashei*	Chilling requirement between 500 and 700 hours Heat, disease, insect and drought tolerant Very productive with firm large fruit Vigorous--3-6m at maturity Small fruit scar Late ripening Adaptive to varied soil type
2N=4X=48	*V. fuscatum*	Evergeen foliage Drought and heat tolerant Low chilling requirement
	V. myrsinites	Evergreen foliage Drought and heat tolerant Low chilling requirement
2N=2X=24	*V. darrowi*	Heat, and drought tolerant Low chilling requirement Evergreen foliage Attractive lowbush form Light blue color
	V. elliottii	Disease resistant Tolerant of mineral soils High berry flavor Vigorous Early ripening
	V. atrococcum	Early ripening Concentrated fruit ripening
	V. arboreum	Tolerant of high pH soils Drought tolerant Late ripening; August through December Native south to Manatee and Hardee Counties, Fl.
	V. staminium	Heat and drought tolerant Low chilling requirement Large fruit

Species hybridization is important in the transfer of desirable traits from diploid to polyploid species (30). Another way to transfer genes from native diploid and hexaploid species into tetraploid highbush, is to produce intermediate tetraploids by interspecific hybridization (43, 57, 80).

Several heteroploid crosses have been made to create introgression bridges with variable results. Hexaploids crossed with diploids generally produce pentaploids when V. darrowi is used as the diploid parent (9, 43). Crosses have also been made between hexaploid V. ashei and diploids V. tenellum and V. elliottii without much success in tetraploid production (31, 43). Hybrids have also been made between hexaploid V. ashei and tetraploid highbush in order to combine favorable traits from each species. These pentaploid interspecific hybrids are readily produced and are vigorous, hardy, and reasonably fertile when pollinated by hexaploids or tetraploids (30, 31). Chromosome elimination during meiosis occurs at a high frequency in blueberry pentaploids and makes them function primarily as tetraploids (58). These interspecific functional tetraploids should cross readily with cultivated highbush to give improved tetraploid breeding lines. This offers one possibility of combining the vigor, productivity, and broad soil adaptability of rabbiteye with the earliness, and quality of highbush (30, 58).

Tissue Culture

Proliferation in tissue culture can occur in 3 ways: enhanced formation of axillary shoots, production of adventitious shoots, and somatic cell embryogenesis (50, 81).

Plant cell culture itself may generate genetic variability and may be employed in some cases to enhance recombination in hybrids. It may also generate variants of commercial cultivars in a high frequency without hybridization to other genotypes (64, 111). One consequence of growth *in vitro* is the appearance of dividing cells with chromosome numbers and karyotypes not usually found in growing points of the intact plant. Aberrant plants are found at rates that approximate mutation rates in field grown plants among plants derived from axillary shoots (101). Aberrations are found at increased rates among plants grown from adventitious shoots, and are even more frequent among plants derived from somatic embryos. When plant cells are allowed to proliferate in callus or suspension culture, there is a high possibility of chromosomal variation. Conditions which favor callus formation have been shown to cause nuclear fragmentation, which produces spontaneous polyploids, aneuploids, and chromosomal rearrangements (10, 85, 86, 88, 98).

Another factor affecting *in vitro* variability is explant source. Murashige and Nakano found constant diploidy to be confined to meristem tissue, while a mixture of diploid and polyploid cells comprised the differentiated

and mature tissues of tobacco. Plantlets derived from regenerated tobacco pith cells were found to vary widely in ploidy level from diploid to octoploid (82).

Adventitious buds are known to form in over 350 species (13, 15). The apex of an adventitious shoot is formed by one or a few vegetative daughter cells of one original cell, thus, its ultimate origin is from a single cell (14). Treatment of explants with mutagenic or polyploidizing agents should result in more whole-plant changes and fewer chimeral changes than are normally encountered following conventional treatments (70), as mutation and adventitious bud formation are both unicellular events. After colchicine treatment, the often less-vigorous polyploid cells are unable to complete with the faster-growing original cells. If a polyploid cell survives, the end product is often a mixoploid or cytochimera. Colchicine treatment in combination with the adventitious bud technique should provide a high percentage of solid, non-cytochimeral polyploids (12, 13, 54).

Adventitious shoot formation has been observed in V. corymbosum, V. ashei, V. atrococcum, V. constablaei, V. darrowi and V. elliottii and their interspecific hybrids. Adventitious shoots and callus have been observed to form from the edges of leaves in contact with the nutrient medium. The callus may, in turn, produce adventitious shoots (71, 84, 112).

The addition of colchicine to *in vitro* systems for induction of polyploidy has been utilized in sugarcane cell suspension (53), daylilly callus (21) and *Hordeum* callus (86). Polyploids of tobacco have been identified phenotypically *in vitro* by their smaller, thicker, greener leaves which were distinctly rosetted and pubescent. Stems were thicker than on the original plants and internodes were shorter. The style and stigma were larger and the ability to produce functional pollen was much reduced. Leaf cells were found to be larger and an additional 2 layers of spongy mesophyll were observed. Chromosomal laggards were often observed in meiotic studies (82).

Radiation Induction of Mutants

Induction of mutants by radiation treatment has its greatest value in vegetatively propagated species in which one or a few characteristics of an otherwise outstanding cultivar may be changed without affecting the remaining genotype (5, 16, 100). Radiosensitivity varies among plant species and depends mainly on the nuclear volume (greater DNA content, more sensitive), chromosome number (plants with fewer large chromosomes are more sensitive than plants with more, smaller chromosomes), and ploidy level (higher ploidy level, less radiosensitive) as well as on the developmental stage of the plant (most resistant seed to dormant plant to highly susceptible growing plant) (16).

Mutation breeding in woody perennials has great promise. The long time lapse between generations, the large space required to grow a plant to maturity in the field, and the high degree of heterozygosity make it difficult to produce cultivars by conventional breeding methods. This is demonstrated by the unusually high ratio of commercial fruit cultivars that have originated from spontaneous bud mutations compared to the cultivars produced by cross breeding (16, 26).

There has been no published work on the radiation induction of mutation in Vaccinium to date. Because some Vaccinium species are heterozygous and diploid, some mutants such as albino or varigated leaves, altered leaf shape, or dwarfed growth form, should be immediately visible following mutagenesis and propagation (16).

Tissue culture should be quite valuable in radiation treatment and mutant identification. Hundreds of bud apexes are contained within a single culture vial and can be treated with ease. Treated shoots, used as explant sources, may regenerate mutant shoots from axillary or adventitious buds. These mutants can easily be screened by visual examination of the in vitro colonies.

Adventitious shoots formed after radiation treatment of irradiated Begonia leaves produced mutants in 30% of the regenerates, 98.5% of which were non-chimeral (14, 90). Tissue culture has also been used successfully in conjunction with radiation treatments with detached leaves of

Achimenes, Saintpaulia, Streptocarpus (13), and Crysanthemum (15).

Gamma radiation has been used in pear to obtain genetically homogeneous shoots by tissue rearrangement of existing periclinal chimeras. Radiation-induced tissue rearrangements have also been used to uncover cryptic mutations hidden within the inner cell layers (32, 41, 42).

CHAPTER III

IDENTIFICATION OF OPTIMAL TREATMENT FOR POLYPLOID INDUCTION

Introduction

In vitro colonies of various clones of *V. elliottii*, *V. darrowi* and their interspecific hybrid (69) were tested to determine optimal explant source, colchicine concentration, treatment duration and mode of treatment for chromosome doubling.

Materials and Methods (Experiments 1-6)

Experiment 1

To determine optimal explant material, 2 types of *V. elliottii* explants from established *in vitro* colonies of Clone 13 were compared. Two-node shoot cuttings were compared with shoot bases which contained scores of axillary buds as explant sources. Colchicine (0.1%) was dissolved in liquid modified Knops medium (103). A wheel drum rotating at about 3 rpm was used to aerate the medium in the treatment vials. Treatment durations tested were 24, 48, 72, and 120 hours. After the treated explants had been rinsed in liquid, colchicine-free modified Knops medium for

1 hour on the wheel drum, five 2-node cuttings were placed in each of 10 new vials containing solid modified Knops medium from each treated vial. After 45 days, the colonies were rated for survival and screened for shoots of increased-diameter, an indicator of increased ploidy (72) (Fig. 1). Leaf casts were made from these shoots and stomate measurements taken.

Shoots of increased diameter screened from the colonies were severed from the shoot bases and rooted in a greenhouse under mist. These subclones were labeled and planted in a field nursery. Leaves and buds were collected from each subclone the following spring for ploidy determination. Five leaves were collected from each subclone and each leaf collected was the third leaf from the apical meristem. Leaf prints were made by brushing fingernail polish on the abaxial leaf surface and removing the cast. The casts were examined microscopically and fifteen stomatal guard cells were measured per leaf cast.

An average of 10 axillary and apical buds were collected from each subclone. After fixing in 1:1 absolute ethanol: glacial acetic acid for 24 hours, the buds were stripped of leaves and softened in an enzyme solution of 0.03 g cellulysin, and 0.03 g pectinase in 5 ml distilled water for 15 minutes. After rinsing, the buds were squashed in a drop of 1% acetocarmine dye. The material was destained with 45% acetic acid to facilitate phase contrast examination of the chromosome number.

Figure 1. Normal diameter *in vitro* shoots of *V. elliottii* (center) and increased diameter colchiploid shoots (left and right).

Experiment 2

To determine optimum treatment duration for treatment with 0.1% colchicine, 2-node cuttings of V. elliottii Clone 10, V. darrowi Clone 5, and the interspecific V. darrowi X V. elliottii hybrid from established in vitro colonies were treated in liquid modified Knops medium on a rotary wheel for durations of 0, 4, 12, 24, and 48 hours. After rinsing, 19 vials were planted from each treatment with three 2-node cuttings on solid modified Knops medium. After 45 days regeneration, the colonies were rated for survival, vigor and increased shoot diameter.

The increased diameter shoots of V. elliottii screened from this experiment were planted on 3 media (6, 67, 103) to determine if shoot production could be enhanced. Vials containing each medium were planted with each of 30 increased diameter subclones. The components of the 3 media used in this experiment are listed on Table 2. Growth response on each medium was recorded 30 days after subculture.

Experiment 3

To determine the optimal colchicine concentration/ treatment duration combination, 2-node cuttings from established in vitro colonies of V. elliottii, V. darrowi and their hybrid were tumbled on a rotary drum (3rpm) in colchicine concentrations of 0.00, 0.01, 0.05, 0.10 or 0.20%

Table 2

Composition of Modified Woody Plant Medium, Modified Andersons Medium, and Modified Knops Medium

Compound	Modified woody plant medium	Modified Andersons medium	Modified Knops medium
	(mg/liter)	(mg/liter)	(mg/liter)
NH_4NO_3	400	400	--
KNO_3	--	480	190
K_2SO_4	990	--	--
KH_2PO_4	170	--	170
$Ca(NO_3) \cdot 4H_2O$	556	--	1,140
$CACl_2 \cdot 2H_2O$	96	440	--
$MgSO_4 \cdot 7H_2O$	370	370	370
$NaH_2PO_4 \cdot H_2O$	--	380	--
Na_2EDTA	74.5	74.5	74.5
$FeSO_4 \cdot 7H_2O$	55.6	55.6	55.6
$MnSo_4 \cdot H_2O$	22.3	16.9	22.3
$ZnSO \cdot 7H_2O$	8.6	8.6	8.6
H_3BO_3	6.2	6.2	6.2
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.25	0.025	0.025
KI	--	0.83	0.83
$CoCl_2 \cdot 6H_2O$	--	0.025	0.025
Pyridoxine . HCl	0.5	--	0.5
Thiamine . CHl	1.0	0.4	0.1
Nicotinic Acid	0.5	--	0.5
Myo-inositol	100	100	100
Adenine Sulfate	--	80	--
Glycine	2.0	--	2.0
Casein Hydrolysate	1,000	1,000	1,000
Sucrose	30,000	30,000	30,000
Agar	4,000	4,000	4,000
2ip	5	5	5

pH adjusted to 5.7 with 1N NaOH. Autoclaved at 1.05 Kg/cm^2 for 15 minutes.

for durations of 6, 12, 24, 48 or 72 hours. A composite of clones was treated for each taxon. For each treatment combination, 10 vials containing modified Knops medium were planted with five 2-node cuttings. Survival, vigor and frequency of increased-diameter shoots were recorded for each taxon after 45 days.

Experiment 4

Two-node cuttings of V. elliottii, V. darrowi and their hybrid were placed on solid modified Knops medium containing 0.00, 0.01, 0.05, 0.10, or 0.20% colchicine to determine optimum colchicine concentration for production of polyploids. After 2, 4, 6, or 8 weeks of treatment, the cuttings were rinsed and replanted on solid modified Knops medium for regeneration. After 45 days, the colonies were evaluated for survival, vigor, and increased-diameter shoots.

Experiment 5

Two-node cuttings from established colonies of 2 clones of V. elliottii (Clones 121 and 133) and 1 clone of V. darrowi (Clone 5) were placed on solid modified Knops medium containing 0.01% colchicine for 3 weeks. The cuttings were rinsed and placed on modified Knops medium. Ten vials were planted from each treated vial. After 60 days the colonies were rated for survival and frequency of increased-diameter shoots.

Experiment 6

Seeds of V. elliottii and V. darrowi collected from open-pollinated wild stands in Florida were placed on solid water/agar medium containing 0.00, 0.10 or 0.25% colchicine to determine seed tolerence and rate of polyploidization. Sixteen vials of each treatment were planted with 5 plump seeds of each species. As soon as germination occurred, the seeds were rinsed and transferred to solid modified Knops medium for colonization.

Results and Discussion (Experiments 1-6)

Experiment 1

Comparison of survival rates for the two explant sources showed a linear decrease in regrowth vigor with increasing duration of colchicine treatment in both treated cuttings and shoot bases. Although the 2 explant types did not differ significantly in survival after treatment, there was a significant difference between explant sources in frequency of shoots with increased-diameter (chi-square probability = .05). The treated cuttings gave rise to 12 vials containing shoots of increased diameter, whereas treated bases produced none. Nine of the 12 vials containing increased-diameter shoots were regenerated from the 48 hour treatment, with the remainder regenerated from the 72 hour treatment. For cuttings, the 48 hour treatment

gave significantly more increased-diameter shoots than treatments of 24, 72 or 120 hours (chi-square probability = .05).

Stomate measurements of in vitro leaves from increased-diameter shoots and normal-diameter shoots were not correlated with stomate measurements of the same cuttings later transplanted to the field nursery. Screening for increased stomate size as an indicator of increased ploidy is, therefore, not recommended at the early in vitro stage.

Stomate guard cell measurements of leaves and chromosome counts of shoot tips from greenhouse rooted thick shoots supported the correlation found earlier between increased stem diameter and induced polyploidy (Figures 2, 3). Seventy percent of the shoots examined had increased stomatal guard cell lengths as well as higher ploidy levels (Table 3). Stomates of Subclone C were significantly larger than diploid or average autotetraploid stomate lengths. This could be due to a periclinal chimera (L-I = 8X? L-III = 4X). If adventitious bud formation were encouraged by in vitro culture of leaves, solid octoploid plants derived from colchicine-treated diploids are possible. Some of the shoots with increased-diameter also showed other morphological anomalies, such as increased and coarse appearing pubesence, darker green, thicker leaves or altered leaf shape. In vitro growth rate was also decreased for large-diameter shoots.

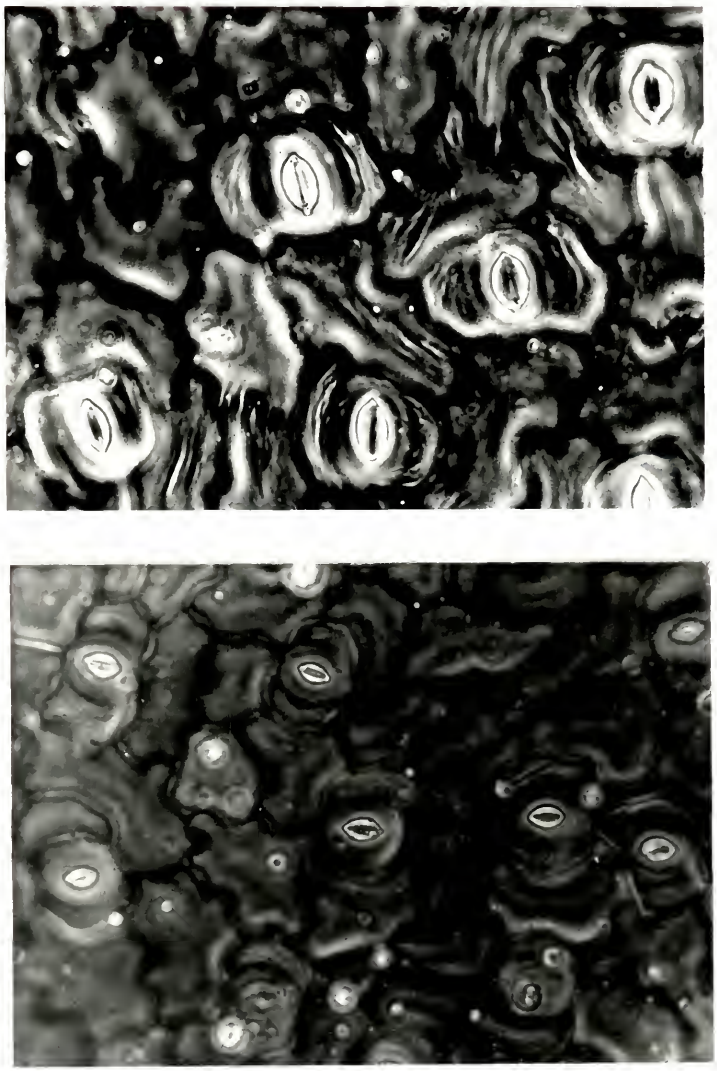

Figure 2. Stomate prints from leaves of an increased-diameter shoot of V. elliottii (top) and a regular-diameter shoot (bottom) photographed at the same magnification.

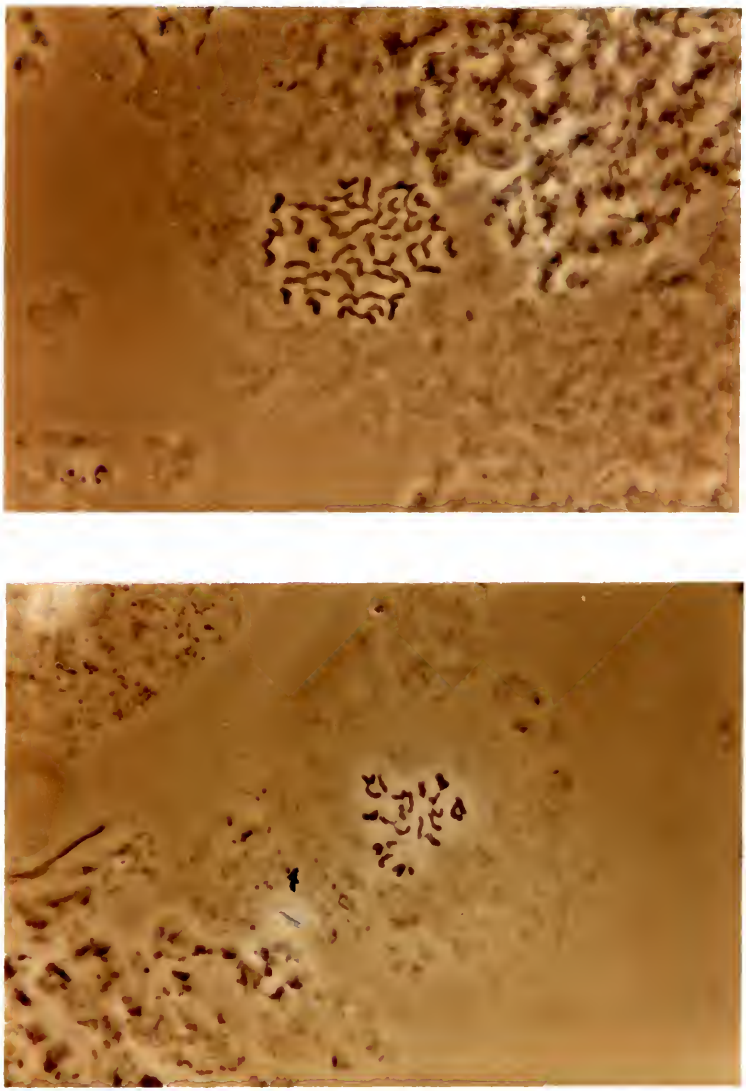

Figure 3. Chromosomes of a shoot tip from an increased-diameter shoot of V. elliottii (2N=ca 48) (top) and an undoubled shoot of regular-diameter (2N=24) (bottom) photographed at the same magnification.

Table 3

Ploidy Level and Stomate Length of 10 *in vitro* Subclones of *V. elliottii* Selected for Large Shoot diameter in Experiment 1

Subclone	Ploidy level[z]	Stomate length (µm)		
		Mean[y]	Range	SD
A	24	104	92-115	11
F	24	115	92-115	15
K	24	118	92-138	12
G	48	172	161-184	12
E	48	173	161-207	12
J	48	181	161-207	14
L	48	184	161-207	15
H	48	187	161-207	12
D	48	189	161-230	15
C	48	240	207-276	14

[z]Counts were determined by shoot tip squashes and are approximate.

[y]Average derived from measurement of 15 stomates per subclone.

Since the treated plant material has yet to flower, assessment of the L-II or gamete-producing layer has yet to be made. Chimeras are common problems in colchicine-treated plant material; therefore, total assessment of increased-diameter shoots is not yet complete. Two of the 3 increased-diameter shoots found to be diploid by chromosome and stomate measurements did flower, and pollen measurements fall within the normal range for diploid V. elliottii.

Aberrant and weak growth was observed in several increased diameter subclone populations following 1 year of growth in the field nursery. Colchicine-induced aneuploidy is a possible explanation for this altered morphology. Vaccinium chromosomes are quite numerous and small, therefore exact counts are not always possible. The chromosome numbers reported in Table 3 are nearest approximations.

Experiment 2

V. elliottii produced both the greatest total number of shoots and the greatest number of shoots of increased-diameter (Table 4). Most of the increased-diameter colonies regenerated from the 4 hour treatment and several from the 12 hour treatment succumbed to fatal decline syndrome (Table 5). Fatal decline is characterized by strong initial growth which ceases when the shoots are 3-5 cm. in height. A few shoots initially take on a water-soaked appearance, and this subsequently spreads throughout the colony. Only colchicine-treated shoots of increased-diameter have been

Table 4

Growth Response of V. elliottii Following Treatment with 0.1% Colchicine for Various Durations

Treatment duration (hrs)	Surviving Shoots			
	Total (no.)	Normal-diameter (no.)	Increased-diameter (no.)	Increased-diameter (%)
0	415	415	0	0
4	410	105	5	1
12	270	181	59	22
24	133	67	66	50
48	135	51	84	62

Chi-square = 337.40*

*Indicates that the ratio of normal-diameter: increased-diameter shoots varied with duration of colchicine treatment.

Table 5

Frequency of Fatal Decline Syndrome Following *in vitro* Treatment of *V. elliottii* with 0.1% Colchicine on a Rotary Drum for Various Durations

Treatment duration (hrs)	Normal-diameter shoots (no.)	Fatal decline shoots (no.)
0	415	0
4	105	300
12	181	30
24	67	0
48	51	0

Chi-square = 643.37*

*Indicates that the ratio of normal-diameter shoots: fatal decline shoots varied with duration of colchicine treatment.

observed to be afflicted. Affected colonies were examined by pathologists, and no fungi or bacteria were found to be associated with the decline symptoms. The decline is hypothesized to be physiological. Perhaps the decline results when some colchicine-induced increased diameter shoots are altered in their response to some medium component. These shoots may be unable to correctly metabolize some nutrient or they may convert some component of the medium into a toxic byproduct.

The 30 increased-diameter subclones of V. elliottii planted on the 3 media varied in growth response and frequency of fatal decline syndrome (Table 6). There is an obvious interaction between media and frequency of production of very vigorous colonies and colonies with fatal decline. Woody plant medium produced both the greatest number of colonies afflicted with fatal decline syndrome and the most very vigorous colonies. Knops and Andersons media were equal in ability to produce vigorous or average colonies. Very few very vigorous or fatally declined colonies were produced on these 2 media.

Increases in callus growth and decreases in shoot production were observed in both V. darrowi (Table 7) and the interspecific hybrid (Table 8) colonies with increased colchicine treatment duration. Callus can originate from leaves, stems, or buds. If the apical and axillary buds succumb to the phytotoxic effect of colchicine, callus

Table 6

Growth Response of Increased-Diameter Shoots of *V. elliottii* when Placed on 3 *in vitro* Media for Regeneration

Medium	Number of colonies which produced growth that was > 20 shoots	10-20 shoots	1-10 shoots
Andersons	3	17	9
Knops	4	27	12
Woody plant	17	3	0

Chi-square = 46.49*

Medium	Number of vials which produced colonies of Healthy growth	Fatal decline syndrome
Andersons	29	3
Knops	43	2
Woody plant	20	16

Chi-square = 23.65**

*Indicates interaction between medium and colony vigor.

**Indicates interaction between medium and frequency of fatal decline syndrome.

Table 7

Growth Response of *V. darrowi* after Treatment of 2-node Cuttings from *in vitro* Colonies with 0.1% Colchicine for Various Durations

Treatment duration (hrs)	Total shoots	Normal-diameter	Increased-diameter
0	169	159	10
4	163	141	15
12	89	83	6
24	52	50	2
48	32	32	0

Chi-square = 4.92 N.S.*

Treatment duration (hrs)	Total vials	Number of vials which produced	
		Callus only	Shoots
0	19	0	19
4	19	1	18
12	19	5	14
24	19	4	15
48	19	11	8

Chi-square = 22.86**

*Indicates non-significant interaction.

**Indicates that the ratio of callus: shoot production varied with duration of colchicine treatment.

Table 8

Growth Response of *V. darrowi* X *V. elliottii* *in vitro* 2-node Cuttings to Treatment with 0.1% Colchicine for Various Durations

Treatment duration (hrs)	Total shoots	Normal-diameter	Increased-diameter
0	102	102	0
4	52	52	0
12	33	33	0
24	27	27	0
48	11	11	0

Treatment duration (hrs)	Total vials	Number of vials which produced	
		Callus only	Shoots
0	19	1	18
4	19	3	16
12	19	6	13
24	19	12	7
48	19	14	5

Chi-square = 28.36*

*Indicates that the ratio of callus: shoot production varied with colchicine treatment duration.

proliferation, characteristic of these taxons in vitro, may still occur.

Experiment 3

Survival rate of explants of the 3 Vaccinium taxons treated with various concentrations of colchicine for various durations in liquid medium on a rotary drum decreased with increasing colchicine concentrations Table 9). V. elliottii had the highest overall survival rating and the highest frequency of increased-diameter shoots (Table 10). Optimal colchicine concentrations and treatment durations for each of the 3 taxons for polyploid induction in this experiment were: V. elliottii--0.01% for 72 hours; V. darrowi--0.01% for 48 hours; V. darrowi X V. elliottii--0.00% for 6 hours. Spontaneous chromosome doubling in V. darrowi and V. darrowi X V. elliottii apparently was responsible for the appearance of large-diameter shoots in the 0.00% colchicine treatments.

Experiment 4

Higher colchicine concentration and longer treatment duration on solid colchicine containing medium were both correlated with decreased survival of 2-node cuttings (Tables 11 and 12). Several regenerated colonies contained shoots of increased diameter. Colchicine concentration of 0.01% for 2-6 weeks was the most successful treatments for production of increased-diameter shoots in V. elliottii.

Table 9

Effects of Colchicine Concentration and Treatment Duration on Regrowth Vigor of Treated *Vaccinium* Explants

Colchicine concn. (%)	Regrowth vigor[z] Duration (hrs) 6	12	24	48	72	Mean
			V. darrowi			
0.00	2.6	1.4	2.1	1.8	2.9	2.2
0.01	2.3	1.2	1.2	1.7	0.9	1.5
0.05	1.7	0.7	1.0	1.6	1.0	1.2
0.10	1.8	0.6	1.0	1.4	0.2	1.0
0.20	1.6	0.1	0.9	1.5	0.1	0.8
Mean	2.0	0.8	1.2	1.6	1.0	

Vigor Score = 1.85 - 5.13 concn.

r = .56**

			V. elliottii			
0.00	3.0	2.2	1.7	2.6	2.1	2.3
0.01	2.4	2.1	2.6	3.1	2.8	2.6
0.05	3.1	1.3	1.3	1.8	0.4	1.6
0.10	2.8	1.7	1.2	1.2	0.2	1.4
0.20	1.4	0.8	0.2	1.7	0.6	0.9
Mean	2.5	1.6	1.4	2.1	1.2	

Vigor Score = 2.66 - 7.61 conc. - .01 hours

r = .68*

Continued

Table 9

Continued

Colchicine concn. (%)	Regrowth vigor[z] Duration (hrs) 6	12	24	48	72	Mean
			V. darrowi x *V. elliottii*			
0.00	2.4	1.7	2.7	1.7	1.6	2.0
0.01	1.8	1.4	2.6	0.8	0.7	1.5
0.05	1.6	2.1	0.7	0.1	0.0	0.9
0.10	1.8	0.8	0.7	0.9	0.0	0.7
0.20	0.7	0.9	0.6	0.9	0.0	0.4
Mean	1.7	1.4	1.5	0.5	0.5	

Vigor Score = 2.21 - 6.78 concn. - .02 hrs.

$r = .81^{**}$

[z]Mean vigor score for 10 vials, each planted with five 2-node explants receiving this treatment (4 = very vigorous; 0 = dead).

[y]In these equations, concn. is expressed in parts per hundred.

*Slope of regression line significantly different from 0.0 at 5% level;

**Slope of regression line different from 0.0 at 1% level.

Table 10

Number of Colonies Containing 1 or More Shoots of Increased-Diameter Following Treatment with Various Colchicine Concentrations for Various Lengths of Time.[z]

Colchicine concn. (%)	Treatment duration (hrs) 6	12	24	48	72	Total
			V. darrowi			
0.00	0	0	0	2	1	3
0.01	0	0	0	3	0	3
0.05	1	0	0	0	0	1
0.10	0	0	0	1	0	1
0.20	0	0	0	0	0	0
Total	1	0	0	6	1	
			V. elliottii			
0.00	0	0	0	0	0	0
0.01	0	0	0	1	7	8
0.05	5	0	0	2	1	8
0.10	1	5	1	2	1	10
0.20	0	0	1	1	1	3
Total	6	5	2	6	10	
			V. darrowi x *V. elliottii*			
0.00	3	1	1	0	0	5
0.01	0	0	2	0	0	2
0.05	0	2	0	0	0	2
0.10	0	1	0	0	0	1
0.20	1	0	0	0	0	1
Total	4	4	3	0	0	

[z]For each treatment, 10 vials were used, each planted with five 2-node cuttings.

Table 11

Effects of Colchicine Concentration and Treatment Duration on Regeneration Vigor of V. elliottii Explants Treated on Solid Modified Knops Medium

Colchicine concn. (%)	Regeneration vigor Treatment duration (wks) 2	4	6	8	Mean
0.00	3.5[z]	3.7	3.9	4.0	3.8
0.01	3.3	1.5	2.5	1.2	2.1
0.05	1.7	0.9	0.7	0.1	0.9
0.10	1.3	0.1	0.4	0.0	0.5
0.20	0.2	0.1	0.0	0.1	0.1
Mean	2.0	1.3	1.5	1.1	

Vigor Score = 3.16 - 14.87 concn. - 0.13 wks.*

[z]Mean vigor score for 10 vials planted with five 2-node explants receiving this treatment (4 = very vigorous; 0 = dead).

*Slope of regression line different from 0.0 at 5% level.

Table 12

Number of *in vitro* Colonies of *V. elliottii* Producing 1 or More Shoots of Increased Diameter 8 Weeks after Planting with Colchicine-treated Explants

Colchicine concn. (%)	No. vials with large diameter shoots[z] Treatment duration (wks) 2	4	6	8	Mean
0.01	6	2	2	0	2.5
0.10	2	0	0	0	0.5
Mean	4	1	1	0	

[z]Of 10 total vials. Five 2-node treated explants were planted per vial.

Experiment 5

The 2 clones of *V. elliottii* and 1 clone of *V. darrowi* treated for 3 weeks with 0.01% colchicine on solid modified Knops medium produced increased-diameter shoots. Three vials of *V. elliottii* Clone 121 and 9 vials of *V. elliottii* Clone 133 contained shoots of increased-diameter. Seven vials of *V. darrowi* Clone 5 were found to contain increased-diameter shoots (Table 13). There was a clonal as well as a species difference in response to colchicine treatment and induction of polyploidy.

Table 13

Growth Response of 3 Vaccinium Clones to Treatment for 3 Weeks on Solid Modified Knops Medium Containing 0.01% Colchicine

Explant source	Total shoots (no.)	Normal-diameter (no.)	Increased-diameter (no.)	Increased-diameter shoots (%)
V. elliottii - Clone 121	70	62	8	11
V. elliottii - Clone 133	290	100	190	65
V. darrowi - Clone 5	41	34	7	19
Chi-square = 87.21*				

*Indicates that the ratio of normal-diameter: increased-diameter shoots varied with the clone treated.

Experiment 6

Although several seeds germinated from each treatment, none of the seedlings which were germinated on medium containing colchicine survived (Table 14). The long time required for seed germination may have allowed high concentrations of colchicine to be absorbed, resulting in phytotoxicity.

Table 14

Response of Seed of V. elliottii and V. darrowi to Colchicine Added to the in vitro Germination Medium

Explant source	Colchicine conc. (%)	Total seed	Number of seed that germinated	Number of seed that survived
V. elliottii	0.00	80	24	24
	0.10	80	7	0
	0.25	80	9	0
V darrowi	0.00	80	26	23
	0.10	80	6	0
	0.25	80	6	0

Conclusions

Tissue culture appeared useful as a vehicle for colchicine treatment. Shoots with increased-diameter could easily be screened by visual examination. The space conservative feature of in vitro culture allowed treatment of a great number of rapidly growing shoot tip cuttings, which enhanced the probability for success. Doubled shoots could be rapidly cloned in vitro allowing large numbers of autotetraploid ramets to be grown. This is quite important because many induced polyploids die before they flower.

The best type of explant to treat is 2-node cuttings. The optimal colchicine concentration/treatment duration combination for induction of polyploids varies with species as well as clone. V. elliottii 2-node cuttings produced the greatest number of increased-diameter shoots when treated for 48 hours on a rotary drum with 0.10% colchicine dissolved in liquid modified Knops medium. When explants were treated on solid modified Knops medium, colchicine at 0.01% for 2-6 weeks was the most successful treatment combination for this species.

Both V. darrowi and V. darrowi X V. elliottii clones produced increased-diameter shoots without addition of colchicine to the treatment medium. Spontaneous doubling exceeded colchicine-induced doubling in the interspecific hybrid and was equal in production of increased-diameter shoots to treatment for 48 hours with 0.01% colchicine in liquid modified Knops medium. Colchicine is apparantly unneccessary for induction of polyploidy in the clones of the 2 taxons tested.

Due to the clonal variation in response to the doubling action of colchicine, it is advisable to treat many clones of a species. Treatment of many clones is also beneficial for obtaining a broad gene base and diverse breeding lines.

CHAPTER IV

ENHANCEMENT OF COLCHICINE ACTION

Introduction

Once optimal *in vitro* colchicine concentration and treatment duration are established for a taxon, polyploids may be easily produced although at a variable frequency. Various researchers have proposed a variety of techniques to reduce the phytotoxicity of the colchicine treatment or to increase the number of cells which are susceptible to the alkaloid.

Materials and Methods (Experiments 7-11)

Experiment 7

DMSO (dimethyl sulfoxide) is used to increase penetration of any substance in solution with it. Colchicine at a concentration of 0.20% was used alone and in combination with 1.0% DMSO in liquid modified Knops medium. Three shoot bases of *V. elliottii* were tumbled on a wheel drum (3 rpm) in each solution for 7 days. The treated shoot bases were rinsed in liquid modified Knops medium for 24 hours and divided to plant 45 vials of each treatment on solid modified Knops medium. After 30 days, the colonies were rated

for survival, vigor and presence of increased-diameter shoots.

Experiment 8

The cytokinin 2iP (6-gamma-gamma-dimethyl-allyl amino purine) encourages axillary bud break and formation of numerous meristems (81). Young, actively growing meristems are known to be ideal material for colchicine treatment. The purpose of this study was to determine the effect of increased levels of 2iP incorporated into the medium both before and after colchicine treatment.

Two-node cuttings of V. elliottii Clone 7 from established in vitro colonies were tumbled in liquid modified Knops medium alone or with 0.1% colchicine. After 48 hours, the cuttings were rinsed and planted on modified Knops medium containing either 5, 20, or 40 mg/l of 2iP. After 8 weeks, the colonies were transferred to modified Knops medium containing 5 mg/l of 2iP.

In addition, various concentrations of 2iP (5, 20 or 40 mg/l) were incorporated into the medium on which seedlings of V. elliottii were planted. The resultant colonies were tumbled in 0.10% colchicine dissolved in modified Knops medium for 48 hours on a rotary drum (3 rpm). The treated colonies were rinsed and planted on solid modified Knops medium containing 5 mg/l of 2iP. After 60 days, the colonies were scored for survival and frequency of increased-diameter shoots.

Experiment 9

Gibberellic acid has been used in combination with etiolation to improve colchicine effectiveness in woody plants (110).

Gibberellic acid concentrations of 10 or 100 ppm were used to treat colonies of V. elliottii Clone 4 with or without a 3 day etiolation period. After 3 days the etiolated colonies were returned to the light and on the fifth day, all vials were treated with 0.10% colchicine for 48 hours on a rotary drum (3 rpm). Eighteen vials were planted from each treatment on solid modified Knops medium. After 45 days, the colonies were rated for survival and frequency of increased-diameter shoots.

Experiment 10

The purpose of this experiment was to determine if colchicine treatment administered before a cold temperature shock would reduce colchicine phytotoxicity or enhance polyploid production. Six shoot bases of V. elliottii Clone 131 were placed in 0.1% colchicine dissolved in liquid modified Knops medium and tumbled on a rotary drum (3 rpm) for 96 hours. After rinsing, the shoot bases were placed on modified Knops medium: 2 at room temperature, 2 in a refrigerator at 7 degrees C. for 48 hours and the remaining 2 at 7 degrees C. for 72 hours. After treatment, each shoot base was divided and 30 vials were planted from each treatment.

After 60 days, the colonies were evaluated for vigor and increased-diameter shoots.

Experiment 11

Auxins are known to induce cell mitosis (44). The purpose of this experiment was to determine the effects of various combinations of colchicine treatment, etiolation and auxin treatment. Several colonies of V. elliottii Clone 10 were etiolated for 3 days, then returned to the light for 1 day. Colchicine at 0.10% was dissolved in liquid modified Knops medium alone or with 1 or 10 mg/l of the auxin, indole-3-butyric acid (IBA), and used to treat 2-node etiolated or non-etiolated cuttings from established in vitro colonies on a rotary drum (3 rpm) for 48 hours. After rinsing, the cuttings were placed on modified Knops medium. Fifteen vials were planted from each treatment combination, and the resultant colonies were rated for vigor and frequency of increased-diameter shoots after 60 days.

Results and Discussion (Experiments 7-11)

Experiment 7

The combination of DMSO and colchicine was quite phytotoxic. Thirty days after treatment, 41 of 45 colonies treated with both colchicine and DMSO were dead and 45 of 45 were dead after 60 days. Twenty-eight of the 45 colonies treated with colchicine alone were dead after 30 days and

32 of 45 were dead after 60 days. Of the surviving 13 colonies, none regenerated shoots of increased-diameter. Although the DMSO did increase the frequency of dead colonies, the high concentration of colchicine (0.20%), the long duration of treatment (7 days) and the use of unresponsive shoot bases as explants all were probably contributing factors to the death rate.

Experiment 8

Growth of colonies on modified Knops medium with 20 ppm 2iP resulted in an increased number of shoots per colony (Table 15). Colony height was decreased with increased cytokinin concentration due to shortened internode length. The 40 ppm 2iP treatment produced a moss-like colony of

Table 15

In vitro Shoot Formation of *V. elliottii* when Cultured on 3 Levels of 2iP in Modified Knops Medium

mg/l of 2iP	Number of shoots[z]
5	57
20	148
40	95

[z]From total of 10 vials per treatment.

shoots. Fewer shoots were apparent per colony because the shoots were so compact that they were indistinguishable from callus.

Due to a high incidence of contamination following colchicine treatment, frequency of polyploid formation could not be meaningfully assessed in this experiment. The increased number of treatable shoot meristems formed with increased concentration of 2iP would seem to favor increased production of polyploids.

Placement of 2-node cuttings following colchicine treatment on media containing various levels of 2iP did not increase the frequency of increased-diameter shoots over what was obtained with 2iP at 5 mg/1. No increased-diameter shoots were detected in the treated colonies in any of the treatments. Since the clones used in this experiment differed from those used in experiments that yielded increased-diameter shoots, possible clonal variation in susceptibility to the polyplodizing effect of colchicine is suggested.

Experiment 9

The use of 0.1% colchicine alone and in combination with 10 ppm or 100 ppm gibberellic acid (G.A.) and/or etiolation in all combinations resulted in increased-diameter shoots in all treatments (Table 16). The non-etiolated, 0 G.A. treatment was as successful in the

Table 16

Frequency of Increased-Diameter *in vitro* *V. elliottii* Shoots Following Treatment with 0.1% Colchicine, Etiolation, and Gibberellic Acid

	Total shoots (no.)	Normal-diameter (no.)	Increased diameter (no.)	Increased-diameter (%)
0 ppm G.A.	54	15	39	72
10 ppm G.A.	41	22	19	46
100 ppm G.A.	95	39	56	59
0 ppm G.A. + etiolation	90	50	40	44
10 ppm G.A. + etiolation	91	51	40	44
100 ppm G.A. + etiolation	118	85	33	28
Chi-square = 36.97*				

*Indicates that the ratio of normal-diameter: increased-diameter shoots varied with treatment.

production of increased diameter shoots as any other treatment combination. Therefore, colchicine treatment of non-etiolated 2-node cuttings is recommended without G.A.

Experiment 10

The cold shock administered after colchicine treatment of *in vitro* shoot bases of *V. elliottii* did not decrease phytotoxicity or increase polyploid induction. Increased refrigeration resulted in increased senescence of shoot bases. No increased-diameter shoots were identified from any of the treatments.

Experiment 11

The combination of etiolation and auxin resulted in increased senescence. The addition of auxin to the treatment medium increased callus formation. After 120 days of regrowth following treatment, the callus had still not regenerated shoots. The cultures were monitored until the medium was depleted, and were then discarded.

Conclusions

None of the enhancement techniques tried was more successful than the standard procedure. Thus, the recommended technique for inducing polyploidy in the *Vaccinium* taxons studied is to treat 2-node cuttings with 0.1% colchicine dissolved in modified Knops medium. The cuttings

should be tumbled on a rotary drum for 48 hours, rinsed, and replanted on solid modified Knops medium for regeneration of colonies.

CHAPTER V

RADIATION STUDIES

Introduction

Mutation induction in vegetatively propagated crops can be valuable in altering a few traits in an otherwise outstanding cultivar. Perennial fruit crops with a long juvenile stage are excellent candidates for this treatment. In order to treat a species most effectively a survey of radiation tolerence must be accomplished.

The purpose of this study was to estimate the LD-50 for *in vitro* gamma irradiation of diploid *V. elliottii* colonies and to screen colonies regenerated from treated cuttings for mutant phenotypes.

Materials and Methods

Twenty colonies of Clone 7 of *V. ellottii* growing on modified Knops medium were subjected to doses of gamma radiation ranging from 0.5 to 50 kilorads (krads). Each vial contained 30-40 miniature shoots, each with 10-15 axillary buds. *V. elliottii* is a highly heterozygous diploid (2N=24) species.

Immediately after treatment, each colony was divided into 10 subcultures of five 2-node cuttings each and

replanted on solid modified Knops medium. After 60 days of regrowth, the colonies were rated for survival.

Results and Discussion

An LD-50 of 4.5 krads was determined for the treated colonies of V. elliottii. The highest dose which produced living colonies was 25 krads. There was a correlation between increasing radiation dosage and decreasing survival (Table 17).

Three colonies (2 from the 1.5 krad treatment and 1 from the 3 krad treatment) appeared to have an altered morphology. In vitro growth of all 3 altered colonies was feathery and dwarfed in appearance compared to the usual small-leaved vigorous growth typical of the other colonies.

Shoots from the altered colonies and from control colonies were severed from the shoot bases and rooted in a greenhouse under mist. About 20 rooted plantlets from each mutant colony and the control were transplanted to a field nursery for observation. The feathery-leaved appearance was not retained in the field. After 1 year in the nursery, differences were noted in leaf color in 1 of the 2 mutants screened from the 1.5 krad treatment. Leaves of this mutant were yellow-green in appearance in contrast with the darker green of normal V. elliottii in the same nursery. Leaves of the mutant became darker green as they matured, but remained paler than leaves of normal plants. The mutant plants were less vigorous than comparable non-mutants, and several died

Table 17

Effect of Gamma Radiation on Regrowth Vigor of *in vitro* *V. elliottii* Explants

Krads	Vigor[z]
0.5	4.0
1.0	4.0
1.5	3.2
2.0	1.0
2.5	2.5
3.0	3.2
3.5	2.6
4.0	1.2
4.5	1.1
5.0	1.7
6.0	0.6
7.0	0.1
8.0	0.0
9.0	0.0
10.0	0.2
15.0	0.2
20.0	0.2
25.0	0.5
30.0	0.0
50.0	0.0

[z]Mean vigor score 10 vials (4 = very vigorous; 0 = dead).

during the first year. The other 2 mutants appeared normal after 1 year of growth in the field.

Conclusions

The combination of *in vitro* plant material and mutagenesis has great potential. Tissue culture colonies contain a high concentration of potentially mutable buds and may also allow mutated cells which would otherwise not survive to be expressed. Once the range of radiation dosage is established for a species, mutations should be easily and readily induced. Only visually apparent mutants were screened from the treated colonies as indicators of optimal dosage. It is recognized that all favorable mutants are not visible.

CHAPTER VI

BREEDING BEHAVIOR OF 12X *V. ASHEI* COLCHIPLOIDS

Introduction

V. ashei (2N=6X=72) and *V. corymbosum* (2N=4X=48) each possess valuable characteristics, and production of the interspecific hybrid could be useful. Direct crosses between the species result in 5X plants which have so far been of little value in further breeding. In hybridizing the two genomes, various chromosome manipulations may be helpful.

Colchicine doubling of 6X *V. ashei* would result in 12X autopolyploids. When crossed with 4X *V. corymbosum*, 8X hybrids might be produced. These 8X's could then be backcrossed to the 4X *corymbosum*, in an effort to produce 6X plants. If this 6X hybrid required more *V. ashei* characteristics, they could be introgressed into the hybrid genome by backcrossing to *V. ashei*.

Materials and Methods

Three doubled plants of *V. ashei* were regenerated from tissue culture treatment with colchicine. Two plants were derived from the cultivar 'Beckyblue', and one from the cultivar 'Bluebelle'. Crosses were made between the 12X

plants using each cultivar both as male and female parent. In addition, the 12X plants were used as male parents in crosses with 6X V. ashei cultivar 'Bonita', 4X V. corymbosum cultivar 'Sharpblue' and 2X V. darrowi. The pollen germination, number of flowers pollinated, fruit set, mean seed number and mean number percent of viable seed were recorded.

The flowers of the 12X plants were larger and thicker than those of their undoubled counterparts (Figure 4). The leaves were larger, darker green and thicker, and the plants were substantially less vigorous than their 6X counterparts.

Pollen of each 12X ramet was germinated using the method of Goldy and Lyrene (49). Freshly collected pollen was dusted on pollen germination medium contained in Petri plates. After 24 hours at room temperature (25 C), the plates were examined under the microscope and percent germination was recorded for 4 fields of vision at 40X magnification.

Results and Discussions

Pollen tube growth of one 12X 'Beckyblue' ramet was strong, while that of the other 12X 'Beckyblue' ramet was weak, with pollen tubes barely protruding from the pollen grain. The 12X 'Bluebelle' clone also had weak pollen tube growth and the pollen clumped in 2 tetrads (Table 18).

No fruit was set when the 12X plants were intercrossed. When used as male parents on 6X V. ashei, only 'Beckyblue'

Figure 4. Flowers from 12X 'Beckyblue' Subclone 1(r.) and from the undoubled 6X 'Beckyblue' (l.) about 1 day before anthesis.

Table 18

Pollen Germination of 12X Colchiploid and 6X *V. ashei* Ramets

	Total grains	Number germinated	% germination
'Beckyblue' I 12X	113	28	25
'Beckyblue' II 12X	181	159	88
'Bluebelle' 12X	270	56	21
Chi-square = 218.30*			
'Beckyblue' 6X	157	144	92
'Bluebelle' 6X	211	186	88

*Indicates that the pollen germination varied with the ramet tested.

subclone I set 4 fruit from 64 pollinated flowers. The mature berries contained many plump seeds. The seeds have not yet germinated, therefore, it is unknown if the seedlings will be true hybrids or accidental outcrosses.

When 'Sharpblue' was crossed with each 12X male parent, many fruit set. Many viable-looking seeds were obtained from the fruit. 'Sharpblue' is highly self-fertile. The high apparent fertility of the 4X . 12X cross seems incompatible with the low degree of pollen viability from the 12X parents, and raises the possibility that the set on 'Sharpblue' was a result of unintentional selfing rather than planned crossing.

A few fruit were set when the 12X males were crossed with 2X V. darrowi females. Very few viable-looking seed were obtained from the berries. Most seeds were flattened and shriveled (Table 19).

Conclusions

The pollen germination and fruit set data suggest that the 12X plants had low fertility both as males and as females.

Table 19

Fertility of 12X *V. ashei* Colchiploids when Crossed with Several *Vaccinium* Species of Various Ploidy Levels and Intercrossed between Cultivars

Female parent	Male parent	# flowers pollinated	# berries harvested	$\bar{x}$ # seed per berry	$\bar{x}$ # seed which appear viable/berry
12X 'Beckyblue' II	12X 'Beckyblue' I	12	0	0	0
12X 'Bluebelle'		10	0	0	0
6X 'Bonita'		64	4	32	1.3
4X 'Sharpblue'		38	21	53	15.8
2X *V. darrowi*		47	0	0	0
6X 'Bonita'	12X 'Beckyblue' II	73	0	0	0
4X 'Sharpblue'		88	26	40	2.4
2X *V. darrowi*		63	3	18	1.0
12X 'Beckyblue'	12X 'Bluebelle'	8	0	0	0
12X 'Beckyblue' II		2	0	0	0
6X 'Bonita'		38	0	0	0
4X 'Sharpblue'		52	17	32	3.8
2X *V. darrowi*		58	1	20	4.0

CHAPTER VII

CONCLUSIONS

Tissue culture is a valuable tool for plant breeding. An *in vitro* colony with its many shoots and numerous buds allows chemical and physical treatments to take place on a large scale with limited space requirements. The supportive environment allows weaker mutated or polyploidized cells to survive and possibly proliferate. The colonies can be easily screened visually for mutants or increased-diameter shoots, a visible indicator of increased ploidy. Once optimum dosage and duration of treatment are established for a species, the numerous polyploidized clones required for introgression of traits into a gene pool can be easily produced.

The most successful polyploidizing treatment varied depending on the taxon and the specific clone treated. For *V. elliottii*, 0.01% colchicine dissolved in liquid modified Knops medium was the most successful medium for polyploid induction. Two-node *in vitro* cuttings treated in this medium for 72 hours on a rotary drum (3 r.p.m.) produced the greatest number of polyploid shoots. Both *V. darrowi* and

V. darrowi X V. elliottii, produced polyploid shoots spontaneously in vitro, without added colchicine. When treatment of 2-node V. elliottii cuttings were treated on solid colchicine containing, modified Knops medium, 0.01% colchicine for 2 weeks duration was the most effective treatment for polyploid induction.

Induced autotetraploids in Vaccinium should be valuable in moving adaptive and economically important traits from diploid native species into cultivated tetraploid breeding lines. In vitro colchicine treatment may also be quite useful in facilitating interspecific gene transfers in other genera where the species differ in ploidy. This will make it possible to tap new gene pools in the breeding of some crop species.

V. elliottii, treated with a wide range of radiation, produced several visible mutants in vitro. An LD-50 of 4.5 krads was established for the treated clone. The mutants, 2 derived from the 1.5 krad treatment and 1 from the 3 krad treatment, were visually identified by altered growth form when compared to control colonies.

Induction of mutations facilitated by radiation treatment of in vitro colonies should be valuable in improvement of long generation, vegetatively propagated perennial crops. Alteration of a few traits of an otherwise outstanding cultivar with mutagenesis may obivate the extensive cycles of hybridization to wild species and backcrossing ordinarily needed to restore the horitcultural value of a cultivar.

The enhancement of adventitious bud formation is an important feature of *in vitro* culture, because it allows formation of a higher frequency of solid polyploids and mutants. In some cases, the *in vitro* system alone is sufficient to allow genetic alterations to occur in explants. With, the added impetus of colchicine or radiation, a much higher frequency of mutant genotypes should be obtained.

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BIOGRAPHICAL SKETCH

Julia Lucile Wood Perry was born on May 6, 1955, in Bethesda, Maryland. She attended high school in Shreveport, Louisiana, at St. Vincent's Academy, graduating in 1973. She attended the University of Colorado, Boulder, for two years, majoring in archaeology. In 1976, she married Robert Thomas Perry. She completed her B.S. degree at Eastern Kentucky University in Richmond in 1977, with a major in horticulture.

She received her M.S. in 1980 from the University of Arkansas, Fayetteville, working under the direction of Dr. James Moore. Her research problem was determination of self- and cross-compatibility of various tetraploid cultivars of blackberry (Rubus Eubatus).

In 1980 she began work with Dr. Paul Lyrene at the University of Florida, Gainesville, to facilitate heteroploid crosses between Vaccinium species by in vitro colchicine treatment of native diploids.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Docotor of Philosophy.

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Paul M. Lyrene, Chairman
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Docotor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Docotor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School, and was accepted as partial fullfillment of the requirements for the degree of Doctor of Philosophy.

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